Long-term elevation of beta-hydroxybutyrate in dairy cows through indusion: effects on feed intake, milk production, and metabolism

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Interpretive Summary:

Long-term elevation of beta-hydroxybutyrate in dairy cows through infusion: effects on feed intake, milk production, and metabolism. *By Zarrin et al*. Effects of an induced hyperketonemia for 48 hours on metabolic variables in plasma and liver of mid-lactating dairy cows were studied. Na-DL-β-OH-butyrate was infused i.v. to obtain plasma beta-hydroxybutyrate (BHBA) concentration above 1.5 mmol/L comparable to a spontaneous hyperketonemia but not caused by a lack of energy and glucose such as in early lactation. Milk yield and feed intake did not change during BHBA infusion. Plasma glucose concentration decreased during the infusion. Declined glucose concentration could not be explained by alterations of insulin or enzymes related to gluconeogenesis, but is likely functionally related to the observed decrease of plasma glucagon concentration.

Long-term elevation of beta-hydroxybutyrate in dairy cows through infusion: effects on feed intake, milk production, and metabolism

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ABSTRACT

Elevation of ketone bodies in dairy cows frequently occurs in early lactation usually concomitantly with a lack of energy and glucose. The objective of the present study was to induce an elevated plasma beta-hydroxybutyrate (BHBA) concentration over 48 hours in mid lactating dairy cows, i.e. during a period of positive energy balance and normal glucose plasma concentrations. Effects of the BHBA infusion on feed intake, metabolism, and performance were investigated. Thirteen cows were randomly assigned to one of two infusion groups, including an intravenous infusion with Na-DL-β-OH-butyrate (1.7 mol/L) to achieve a plasma concentration of 1.5 to 2.0 mmol/L of BHBA (HyperB, n=5), or an infusion of 0.9 % saline solution (control, n=8). Blood was sampled before and hourly during the 48 h of infusion. In liver, mRNA transcripts related to gluconeogenesis (pyruvate carboxylase (PC), glucose 6-phosphatase (G6PC), mitochondrial phosphoenolpyruvate carboxykinase (PEPCKm)), Phosphofructokinase (PFKL), pyruvatedehydrogenase complex, and fatty acid synthesis (acetyl-CoA carboxylase (ACoAC), fatty acid synthase (FASN)) were measured by real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin were used as housekeeping genes. Changes (difference between before and after 48h infusion) during the infusion period were evaluated by analysis of variance with treatment as fixed effect, and area under the curve (AUC) of variables was calculated on the second day of experiment. The plasma BHBA concentration in HyperB cows was 1.74 ± 0.02 mmol/L

(mean ± SE) compared with 0.59 ± 0.02 mmol/L for control cows. The change in feed intake, milk yield and energy corrected milk (ECM) did not differ between the two experimental groups. Infusion of BHBA reduced the plasma glucose concentration (3.47 ± 0.11 mmol/L) in HyperB compared with control cows (4.11 ± 0.08 mmol/L). Plasma glucagon concentration in HyperB was lower than the control group. All other variables measured in plasma were not affected by treatment. In the liver, changes in mRNA abundance for the selected genes were similar between two groups. Results demonstrate that intravenous infusion of BHBA decreased plasma glucose concentration in dairy cows but this decrease could not be explained by alterations in insulin concentrations or key enzymes related to gluconeogenesis. Declined glucose concentration is likely functionally related to decreased plasma glucagon concentration.

Key words: dairy cow, ketone bodies, glucose, beta-hydroxybutyrate, metabolism

INTRODUCTION

High yielding dairy cows need a lot of energy and nutrients for maintenance and milk synthesis in particular during peak lactation. Despite of increased feed intake in the early lactation (Agenäs et al., 2003) after the periparturient nadir, this adaptation cannot cover the requirements during this period. Typical metabolic changes during this period of negative energy balance are low plasma concentrations of glucose, and high concentrations of plasma non-esterified fatty acid (NEFA) and subsequently elevation of ketone bodies (van Dorland et al., 2009; Gross et al., 2011). Although ketone bodies can be used as an alternative fuel for some tissues such as brain and heart (Laffel, 1999; Veech, 2004), kidney (Weidemann and Krebs, 1969), skeletal muscles (Ruderman and Goodman, 1973) and lactating mammary gland (Shaw, 1943), the utilization of ketone bodies is limited. Unused ketone bodies

concentration increase tremendously in blood (Duffield et al., 2009). This elevation of ketone bodies in blood causes reduced feed intake and increases the risk of clinical ketosis (CK), displaced abomasums (DA), metritis and subsequent decrease of milk production (Duffield et al, 2009). The mostly used method to detect of subclinical ketosis is the measurement of BHBA in serum or plasma (Duffield, 2000; Herdt, 2000). In ruminants, BHBA is the major ketone body found in circulation (Bergman, 1971). Ospina et al (2010) suggested a threshold value of 1200 µmol/L to distinguish between normal cows and subclinical ketotic cows.

Subcutaneous BHBA infusion in rats (Langhans et al., 1983; Moor et al., 1976), intraperitoneal BHBA infusion in goats (Rossi et al., 2000), and intracerebroventricular BHBA infusion in dairy cows (Kuhla et al., 2011) lead to inhibited feed intake in these animals. Intravenous BHBA infusion lead to decreased plasma concentration of glucose in dogs (Madison et al., 1964), pigs (Müller et al., 1984), and ewes (Schlumbohm and Harmeyer, 2003, 2004). The reason of declined glucose during the infusion of BHBA remained unclear, but authors suggested that infusion of BHBA induced hypoglycemia through direct or indirect inhibition of gluconeogenesis or by change in insulin concentration in pig and sheep (Müller et al., 1984; Schlumbohm and Harmeyer, 2004). This mechanism has not yet been investigated in dairy cows. Our objective was to induce an elevated plasma BHBA concentration over 48 hours and to investigate its effects on feed intake, performance, and metabolism in the dairy cow. More specifically, this study was carried out to confirm that also in the dairy cow, like in sheep and pig, elevated plasma BHBA concentration affects plasma glucose concentration, and to investigate the underlying mechanisms.

MATERIAL AND METHODS

Animals and Management

100 The present trials followed the Swiss Law on Animal Protection and were permitted by the
101 Committee of Animal Experiments of the Canton Fribourg, Switzerland. The study was
102 carried out with 13 diestrus Holstein dairy cows with a parity of 3.5 ± 0.10, at 28 ± 0.3
103 (MEAN ± SD) weeks in milk. Cows in later lactation were selected to allow the investigation
104 of the specific effect of BHBA infusion without influences of the characteristic endocrine and
105 metabolic changes during the transition period. The cows were healthy at the start of the
106 experiment which was confirmed by a routine blood glutaraldehyde coagulation test
107 (Sandholm, 1976), and by measuring milk somatic cell count (DeLaval cell counter DCC,
108 Switzerland), which had to be less than 150,000 cells/mL in all four quarters.
109 Two weeks before the start of the experiment, animals were allowed to adapt to tie stall
110 housing and to the experimental feeding conditions. Animals were fed ad libitum with hay. In
111 addition**,** cows were fed a protein- and energy- rich concentrate (Barley 23.5%, Oats 14.0%,
112 Wheat bran 20.0%, Expeller soybean 17.0%, Linseed meal 15.0%, Salt livestock 0.6%,
113 Carbonate of lime 2.2%, KAF premix 0.4%, Molasses 4.0%, and Alikon 3.0%) twice daily
114 according to each cow's individual extra energy requirement for milk production**,** and once
115 daily 50 g of minerals. Water was available ad libitum. Milking was performed twice daily at
116 0530 h and 1600 h.
117
118 ***Experimental design and treatments***
119 The thirteen cows were randomly assigned to one of two infusion treatments. The treatments
120 included an infusion with BHBA (HyperB, n=5) to obtain a plasma BHBA concentration
121 between 1.5 to 2.0 mmol/L. The control treatment (n=8) included an infusion with a 0.9 %
122 saline solution (NaCl, 20 mL/h). One day before the infusions started, cows were fitted with
123 indwelling intravenous catheters (Cavafix® Certo® Splittocan®, B. Braun Melsung AG,
124 Germany) with a length of 32 cm and a diameter of 16 G in both jugular veins. The infusions
125 through one of the catheters started at 9:00 am and continued until 09:00 am two days later.

126 The initial infusion rate (8.5 ± 0.6 µmol/kg/min) was determined on the basis of the body
127 weight of each animal and the molarity of the solution followed by instant continued
128 adjustments throughout the 48 h to maintain a plasma BHBA concentration between 1.5 to 2.0
129 mmol/L by a peristaltic pump (Perpex Jubile pump, H. J. Guldener, Zürich, Switzerland).
130 During the first two hours of BHBA infusion, the blood samples were taken every 15 min to
131 measure the plasma BHBA concentration and adjust the BHBA infusion rate to reach the
132 plasma BHBA concentration between 1.5 and 2.0 mmol/L.
133 The control cows were treated with 0.9 % saline solution at an infusion rate of 20 mL/h
134 administered using an automatic pump (Perfuser®, B. Braun Melsung AG, Germany).
135
136 ***Preparation of the BHBA solution***
137 To achieve a 1.7 M BHBA solution 279.053 g of DL-Beta-hydroxybutyric acid sodium salt
138 (Sigma-Aldrich, H6501) was dissolved in bidistilled water to reach an amount of 1.0 L
139 followed by homogenization using a rotor mixer during 10-20min. The pH of the solution was
140 adjusted with HCl to 7.4 and sterilized at 1 bar and 131ºC for 50 min. The solution was
141 filtered through a 0.2µm ø filter (Millipore Express® PLUS Membrane 45 mm, Millipore,
142 China) and stored at 4°C until infusion.
143
144 ***Data collection and sampling***
145 ***Feed and milk samples***. The individual intake of hay was recorded daily for each cow by
146 weighing the amount given and subtracting it by the remainder of hay found the next
147 morning. The milk yield was recorded for each cow individually at every milking during the
148 adaptation and experimental period. Proportional milk samples were taken during milking in
149 the afternoon and in the morning before the start of the experimental infusions. The milk
150 sampling was repeated in the afternoon of day two (32 h after the start of infusion), and at the
151 morning milking at the end of experiment.

Blood samples. One week before the experiment, and immediately before the start of the experimental infusions on day 1, reference blood samples of each cow were taken from a jugular vein via vacuum tubes containing tri-potassium-EDTA at 0730 h, after milking and before feeding. The tubes were kept on wet ice until they were centrifuged for 20 minutes at 3000 x *g*, and the plasma was obtained. Until the samples were analyzed for concentrations and activities of plasma variables, the plasma was frozen at -20 °C. After analysis, the mean value of these samples was calculated and used as reference concentration in the statistical evaluation. During the experimental infusions blood samples were obtained from the contralateral jugular catheter, which was not used for the infusion, by using tubes containing tri-potassium-EDTA**.** The collected blood samples were partly analyzed immediately for BHBA concentration (described under laboratory procedures) as a basis for adjustments of the infusion rate, or were handled similarly as the reference samples.

Liver biopsies. One week before the infusions and 48 h after the start of the infusions, following the blood sampling, at around 0800 h, a liver biopsy was taken. Liver tissue (60 to 100 mg) was obtained under local anesthesia with 10 mL Lidocain 2% (Streuli Pharma AG, Uznach) by blind liver puncture with a 14 G and 150 mm or 200 mm long biopsy needle (Tru-Cut, Provet AG, Lyssach, Switzerland). The samples were placed directly into RNA stabilization reagent (RNAlater®, Ambion, Applied Biosysthems, Austin, TX), kept at +5 °C for 24 hours and thereafter stored at –80 °C until RNA extraction.

Laboratory procedures

Feed and milk. The nutritional composition of hay (DM content, 890g/kg of fresh matter (FM); on DM basis, consisting of 153 g of CP/kg, 235.0 g of Crude fiber/kg, and 5.7 MJ of NE_L/kg) and concentrate (DM content, 881 g/kg of FM; on DM basis, consisting of 217 g of

178 CP/kg, 73.9 g of Crude fiber/kg, and 7.6 MJ of NE_L/kg) were determined by using routine
179 analyses as described by van Dorland et al. (2009). The nutritive energy balance was
180 calculated as the difference between the energy intake, and the energy output (NE_L,
181 requirements for maintenance and milk production, determined from tabulated values and
182 recommended equations according as to van Dorland et al. (2009)).
183 Milk samples were analyzed at the laboratory of the Holstein Association of Switzerland,
184 Grangeneuve, Switzerland, for their content of fat, protein, lactose and urea (CombiFoss
185 6000, Gerber Instrument AG, Effretikon, Switzerland). The ECM was calculated based on
186 Sjaunja et al. (1991), using the formula: ECM (kg/d) = [(0.038 × Fat (g/kg) + 0.024 × Protein
187 (g/kg) + 0.017 × Lactose (g/kg)) × Milk Yield (kg/d)] / 3.14
188

189 ***Blood plasma.*** Plasma metabolites were measured enzymatically with an automated analyzer
190 (Cobas Mira 2, Hoffmann-La Roche, Basle, Switzerland) by the use of commercial kits as
191 described by van Dorland et al. (2009) and Kreipe et al. (2011). Plasma insulin and insulin-
192 like growth factor-1 (IGF-1) were measured by RIA as described by Vicari et al. (2008) and
193 total cortisol was measured by RIA as described by Blum et al. (1985). Plasma glucagon
194 concentration was measured by using a RIA kit (cat. # GL-32K, MILLIPORE, Zug,
195 Switzerland).
196

197 ***Liver tissue.*** Total mRNA was extracted from liver samples using peqGOLD TriFast™
198 (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to manufacturer's protocol,
199 and afterwards quantity and purity were determined by absorbance at 260 nm and 280 nm
200 using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington,
201 DE). Subsequently, one µg of total mRNA was reverse transcribed with 200 U Moleney
202 Murine Leukemia Virus Reverse Transcriptase RNAase H Minus, Point Mutant (Promega
203 Corporation, Madison, WI) using 100 pmol random hexamer primers (Invitrogen, Leek, The

Netherlands). A master mix containing the following components was prepared: 0.8 µL PCR water, 1.0 µL forward primer (5 pmol), 1.0 µL reverse primer (5 pmol), 5.2 µL 2x SensiMix plus SYBR-Green (1 mM MgCl2). The selected and measured genes encoding for hepatic enzymes involved in gluconeogensis, glycolysis, fatty acid oxidation, citrate synthase, fatty acid and triglyceride synthesis, cholesterol synthesis, pyruvate dehydrogenase complex, and ketogensis, are shown in Table 1. The primer sequences for PC and PEPCKm were according to Hammon et al. (2003), FASN, acyl-CoA synthetase long-chain (**ASCL**), carnitine palmitoyltransferase 1A (**CPT1A**), and citrate synthase (**CS**) were according to van Dorland et al. (2009), ACoC, carnitine palmitoyltransferase 2 (**CPT2**), G6PC, and 3- hydroxyl-3 – methylglutaryl-coenzyme A synthase 1 (**HMGCS1**), according to Graber et al. (2010). Lactate dehydrogenase A (**LDHA**), lactate dehydrogenase B (**LDHB**), PFKL, dihydrolipoamide branched chain transacylase E2 (**DBT**), dihydrolipoamide dehydrogenase (**DLD**), pyruvatedehydrogenase (lipomide) alpha 1 (**PDHA1**), glucagon receptor (**GCGR**), glycogen synthase 2 (**GYS2**), and protein kinase, AMP- activated alpha 1 (**PRKAA1**) primers were designed to amplify cDNA.

The PCR quantification was performed by using a Rotor-Gene™ 6000 (Corbett Research, Sydney, Australia), using the software version 1.7.40. Fluorescence take off was calculated with the "threshold" program option. Eight µL of master-mix and two µL sample volume, containing 20 ng of cDNA, were used. The following three-step PCR program was performed: denaturation for 10 min at 95 °C, 40 cycles of amplification (each consisting of 15 s at 95 °C, the primer-specific annealing temperature for 30 s (Graber et al., 2010; Pfaffl et al., 2002), and extension at 72 °C for 20 s and quantification of fluorescence, and finally a melting curve program (60 to 95 °C). The mRNA levels were calculated relative to the mean expression level of the housekeeping genes GAPDH and ubiquitin, which were stable across time points as described by Kreipe et al (2011).

230 ***Statistical analysis***

231 Prior to data analysis, for each variable (gene expression, plasma metabolites, feed intake,
232 milk yield, and ECM), the change (delta) was calculated by subtracting the value measured
233 after the infusion treatments from the value measured before the infusion treatments. The
234 delta for each variable (difference between before and after 48h infusion) was statistically
235 evaluated using the general linear models (GLM) procedure of SAS (SAS Institute Inc., Cary,
236 NC, USA, 2002-2008, Release 9.2), including treatment (BHBA or NaCl) as fixed effect.
237 Differences between means were determined by the Tukey test. In addition, means of delta
238 values obtained within each treatment were tested for their difference from "0". Furthermore,
239 blood plasma variables, the area under the curve (AUC) was calculated for day 2 (d2) of the
240 treatments, and subsequently assessed statistically with the model as described above. Data
241 obtained prior to the treatment were included as covariate in the model to compensate for
242 initial differences between individuals.

243 Data are presented as means ± SEM and differences were considered significant if $P < 0.05$,
244 and as a trend if $P < 0.10$.

245

246 **RESULTS**

247 ***Infusion rates and plasma concentrations of BHBA***

248 The mean infusion rate of BHBA over 48 h was 8.5 ± 0.6 µmol/kg/min (Figure1). The mean
249 plasma BHBA concentration reached and maintained was 1.7 ± 0.1 mmol/L for 48 h.

250 The infusion of BHBA resulted in a marked increase in plasma BHBA concentration in
251 HyperB cows ($P < 0.001$). Plasma BHBA concentration in control cows remained unchanged.
252 Consequently, larger AUC was observed (Table 2) for plasma BHBA concentration in
253 HyperB (1.74 ± 0.02 mmol/L, mean ± SEM) cows compared with the control cows (0.59 ±
254 0.02 mmol/L) on d2 ($P < 0.001$).

255

256 ***Feed intake, energy balance, and ECM***

257 Dry matter intake (DMI) of hay and concentrate prior to the infusions (d0) is shown in table 3.
258 On day 1 of the infusions, DMI of hay and concentrate was 19.1 ± 0.4 kg/d, and 16.9 ± 1.9
259 kg/d, for HyperB, and control cows, respectively. On day 2 of the infusions, DMI of hay and
260 concentrate was 19.0 ± 0.9 kg/d, and 17.9 ± 1.0 kg/d, for HyperB, and control cows,
261 respectively. The DMI was not affected by BHBA infusion.

262 The nutritive energy balance was remained unaffected by HyperB and similar in both groups
263 across the study period. The change from d0 to d2 (delta), was -1.04 ± 8.67MJ/d, and 1.72 ±
264 4.9 MJ/d for HyperB and control cows, respectively. Milk yield and ECM were not affected
265 by BHBA infusion.

266

267 ***Blood metabolites and enzymes***

268 Plasma glucose concentration in HyperB cows decreased (P = 0.03, Table 4) and was lower
269 compared with control group on d2 (P = 0.0012, Table 2) (Figure 2). Beta-hydroxybutyrate
270 infusion decreased the plasma glucagon concentration on day 2 (P = 0.03) (Figure 3). Plasma
271 concentrations of NEFA, IGF-1, Urea, triglyceride (TG), Cortisol, and Insulin (Figure 4)
272 remained unchanged in both HyperB and control groups during the course of infusion. The
273 plasma Urea, IGF-1, and TG decreased within control group, and the plasma glucose and urea
274 decreased within HyperB group.

275

276 ***Liver gene expression***

277 The mRNA abundance in liver of the most studied variables did not differ between treatments
278 (Table 5). Beta-hydroxybutyrate infusion affected on mRNA abundance of PRKAA1, and it
279 tended to lower in HyperB group than the control group (P=0.08). The mRNA abundance of
280 PC increase and that of PDHA1, and HMGCS1 decreased (P<0.05) over time within the

281 control cows. In the HyperB group the mRNA abundance of HMGCS1 decreased
282 significantly over time.
283
284 **DISCUSSION**
285 Our objective was to induce an elevated plasma concentration of BHBA without the
286 metabolic situation as observed during the early lactation, to solely investigate the effect of
287 BHBA on feed intake, milk yield, and metabolism. This study is to the best of our knowledge
288 the first one that induced an elevated plasma BHBA concentration for 48 h in dairy cows by
289 infusion of BHBA. Plasma BHBA concentration reached was 1.7 ± 0.1 mmol/L, which was
290 clearly above the mostly considered concentration of 1.2 to 1.4 mmol/L referring to
291 subclinical ketosis (Duffield et al., 2009; Ospina et al., 2010).
292 The expected reduction of DMI in HyperB cows as shown in cows with spontaneously
293 elevated ketone body concentrations (Bareille et al., 2003; Gonzàlez et al., 2008) was not
294 observed in our study. Subcutaneous injection of BHBA (10 mmol/kg body weight) in rats
295 (Langhans et al., 1983; Moor et al., 1976), intraperitoneally BHBA infusion (15 mmol/$kg^{0.75}$)
296 in pigmy goats (Rossi et al., 2000), and intracerebroventricular BHBA infusion (18mM/d) in
297 dairy cow (Kohla, 2011) decreased feed intake. Beta-hydroxybutyrate can play a role in
298 regulation of feed intake (Langhans, 1983). In an *in vitro* experiment, elevation of BHBA:
299 glucose ratio to 1:1 in early lactation compare with non-ketotic cows with a 1:4 ratio, can
300 decrease feed intake via stimulation of dephosphorylation of AMPK after parturition (Laeger
301 et al, 2012). In our study, BHBA infusion did not affect feed intake throughout the experiment
302 which may relate to low ratio of BHBA: glucose.

303 During the experiment plasma glucose concentration decreased dramatically due to the
304 infusion of BHBA. This finding is consistent with other reports of decreased plasma glucose
305 concentration associated with elevated BHBA in pregnant sheep (Schlumbohm and Harmyer

306 2003, 2004), dogs (Madison et al., 1964; Felts et al., 1964), and pigs (Müller et al, 1984).
307 However, our findings are in contrast to other reports that described a hyperglycemia in
308 rabbits (Mirsky and Broh- Kahn, 1937; Caccuri, 1937) induced by ketone bodies infusion.

309 Information about the reasons for decreased plasma glucose concentration due to the elevated
310 BHBA concentration is limited. Hypothetically, insulin plays a role in this effect. Infusion of
311 BHBA increases insulin secretion in pigs (Müller 1984). Madison et al (1964) suggested that
312 pancreatic beta cells respond to the high plasma BHBA concentration with increased insulin
313 secretion in dogs. It appears that insulin suppresses the glucose production throughout an
314 inhibitory effect on the regulatory enzymes of gluconeogenesis (Hayirli, 2006; Brockman and
315 Larveld, 1986). This release of endogenous insulin can lead to decline in plasma glucose
316 concentration, and reduce the hepatic glucose production. The unchanged insulin
317 concentration in response to the BHBA infusion in the present study may be explained by the
318 fact that ketone bodies represent only a modest stimulus on insulin secretion in ruminant
319 (Jordan and Philips, 1978; Heitman and Fernandez, 1986). Insulin did obviously not play a
320 significant role in mediating the lowering effect of BHBA on plasma glucose concentration in
321 sheep (Schlumbohm and Harmyer 2003). Decreased glucose concentration and hepatic
322 glucose output due to the infusion of BHBA can reduce the availability of glucose for the
323 peripheral tissues because exhausted glucose stores must be replaced by a catabolism of
324 proteins during emergency conditions for the vital tissues such as central nervous system
325 (Madison et al., 1964). The molarity of ketone bodies in plasma are the same or higher than
326 that of glucose. Thus, enhanced plasma BHBA concentration can increase the efficiency of
327 BHBA as a competitor to glucose used by peripheral tissues and has been shown to inhibit
328 peripheral glucose utilization (Madison et al., 1964). Ketone bodies are a preferred energy
329 source by body tissues compared to glucose, because 100 g of BHBA produces 10.5 kg ATP
330 whereas 100 g glucose produces 8.7 kg ATP (Salway 1999). Mebane et al (1962)

demonstrated that ketone body infusion leads to a 30% reduction in glucose utilization by peripheral tissues. Previous studies also reported that the oxidation of glucose was inhibited by ketone body infusion (Neptune et al., 1961; Williamson and Krebs, 1961). In contrast, as above mentioned Schlumbohm and Harmyer (2004) suggested that BHBA infusion decreased endogenous glucose production but did not affect glucose utilization in pregnant sheep. Furthermore they suggested that the elevated concentration of ketone bodies in blood initially decline glucose production via the depressed signal on hepatic gluconeogenesis in liver.

In contrast to a previous study that found increased glucagon concentrations after BHBA infusion in pigs (Müller 1984), in our study BHBA infusion decreased glucagon concentration in HyperB compared with the control group. Plasma glucose is derived from the feed, glycogen, and by gluconeogenesis, and it is regulated by several hormones, mainly glucagon and insulin. Glucagon increases plasma glucose concentration through stimulation of gluconeogenesis and glycogenolysis (Aronoff et al, 2004; Grald and Taborsky, 2010). Change in glucagon secretion from the physiological range results in changes of plasma glucose concentration (Grald and Taborsky, 2010). Some substrates such as NEFA and ketone bodies suppress glucagon secretion (Gerich et al., 1974; Gerich et al., 1976; Goberna et al., 1974). There is evidence that glucagon secretion is inhibited by the neighboring β- cell through insulin (Weir et al, 1976) and gamma amino butyric acid (GABA) (Adeghate et al., 2000; Wendt et al., 2004). Gamma amino butyric acid is an inhibitory neurotransmitter in the brain. In an in vitro study it was demonstrated that BHBA increase GABA concentration in the epileptic brain (Suzuki et al., 2009). We suspect that the decrease in glucagon concentration in our study is related to elevated BHBA concentration as BHBA is used as an alternative fuel and energy source for the tissues, or glucagon secretion was prevented by the GABA inhibitory effect. Subsequently a decline of glucagon secretion decreased glucose production through the effect on glycogenolysis or gluconeogenesis during the experiment.

356 We have expected a decrease of plasma NEFA concentration in response to the BHBA
357 infusion and a subsequent increase of insulin (Madison et al., 1964), both variables did not
358 change during the experimental period.
359 Beta-hydroxybutyrate can replace glucose to be utilized in peripheral tissues (Madison et al.,
360 1964), and this can help to decline fatty acid mobilization from adipose tissues. On the other
361 hand after a decline in glucose concentration and subsequently NEB, enhance of NEFA
362 occurred in early lactation (van Dorland et al., 2009), but in this study despite of decreased
363 glucose, cows were not in the NEB situation. Although liver is the main site to produce
364 ketone bodies from fatty acid oxidation, it cannot utilize the ketone bodies for the synthesis of
365 acetoacetyl CoA and subsequently fatty acids due to a lack of the Succinyl-CoA:3-ketoacid-
366 coenzyme A transferase 1, mitochondrial (OXCT1), (Goodridge and Sul,, 2000).
367
368 ***Effects of BHBA infusion on hepatic gene expression***
369 Our results of mRNA abundance of key enzymes related to hepatic gluconeogenesis (pyruvate
370 carboxylase, glucose-6-phosphatase, mitochondrial phosphoenolpyruvate carboxykinase) are
371 in contrast with suggestions from previous studies that indicated an inhibition of
372 gluconeogenesis by elevated plasma BHBA concentration (Schlumbohm and Harmyer, 2003;
373 Müller 1984). Müller et al (1984) suggested that ketone bodies probably have a direct
374 inhibitory action on gluconeogensis. They mentioned that the effect of ketone bodies on
375 endogenous glucose production is probably mediated by insulin. However, in ruminants the
376 effect of ketone bodies on insulin secretion is not strong (Jordan and Philips, 1978; Heitman
377 and Fernandez, 1986), and insulin concentration did not change in our study. Soling and
378 Kleineke (1976) suggested that ketone bodies have a glucose sparing effect in tissues through
379 mitochondrial formation of citrate, an allosteric inhibitor, phosphofructo-1,6-kinase, acetyl-
380 CoA inhibition of pyruvate dehydrogenase and activation of pyruvate carboxylase. Beta-
381 hydroxybutyrate infusion did not affect on key enzymes related to gluconeogensis, glycolysis,

382 pyruvate dehydrogenase complex (PDH complex), and citrate synthase mRNA expression.
383 According to the mRNA abundance of some enzymes related to fatty acid oxidation, BHBA
384 infusion did not affect fatty acid oxidation. These results are not in agreement with previous
385 reports (Soling and Kleineke, 1976; Morio and Wolfe, 2005) that mentioned ketone bodies are
386 an inhibitor for fatty acid oxidation through increase acetyl CoA production, inhibit CPT1
387 activity, and stimulate malonyl CoA synthesis.

388 As mentioned above ketone bodies suppressed the glucagon secretion, and this inhibition
389 could have been regulated via cAMP. The mechanism of this inhibition is related to the
390 generation of ATP/ADP by substrate such as ketone bodies, then decrease of cAMP and
391 finally prevention of glucagon secretion (Gerich et al., 1976; Toyota et al., 1975; Mitrakou et
392 al., 1991; Adeghate et al., 2000). In the present study, the PRKAA1 expression tended to
393 decrease in HyperB compared with control group (P<0.1). On the other hand, BHBA infusion
394 decreased the PRKAA1 expression, subsequently glucagon excretion was decreased.

395

396 **CONCLUSION**

397 Results from this study demonstrate that intravenous BHBA infusion successfully induced
398 elevated plasma BHBA concentration for a long term (48 h) in dairy cows. Based on this
399 study feed intake is not affected by elevated BHBA concentration during a period positive
400 energy balance. Decreased plasma glucose concentration during BHBA administration cannot
401 be related to changes of insulin concentration or to mRNA abundance of genes related to
402 hepatic gluconeogenesis, fatty acid synthesis, citrate synthase, pyruvate dehydrogenase
403 complex, and glycolysis. Although mRNA expression indicated high activity of enzyme
404 production, the effects of BHBA infusion occurred most likely at a post-transcriptional level.
405 Reduced glucose concentration maybe related to decrease in plasma glucagon concentration

406 which can decrease glucose production from gluconeogenesis or glycogen storage. Thus
407 BHBA seems to have a glucose sparing effect. However, glucose entry rate was probably
408 reduced by the treatment, and additional studies will be necessary to clarify the underlying
409 mechanisms.

410

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416

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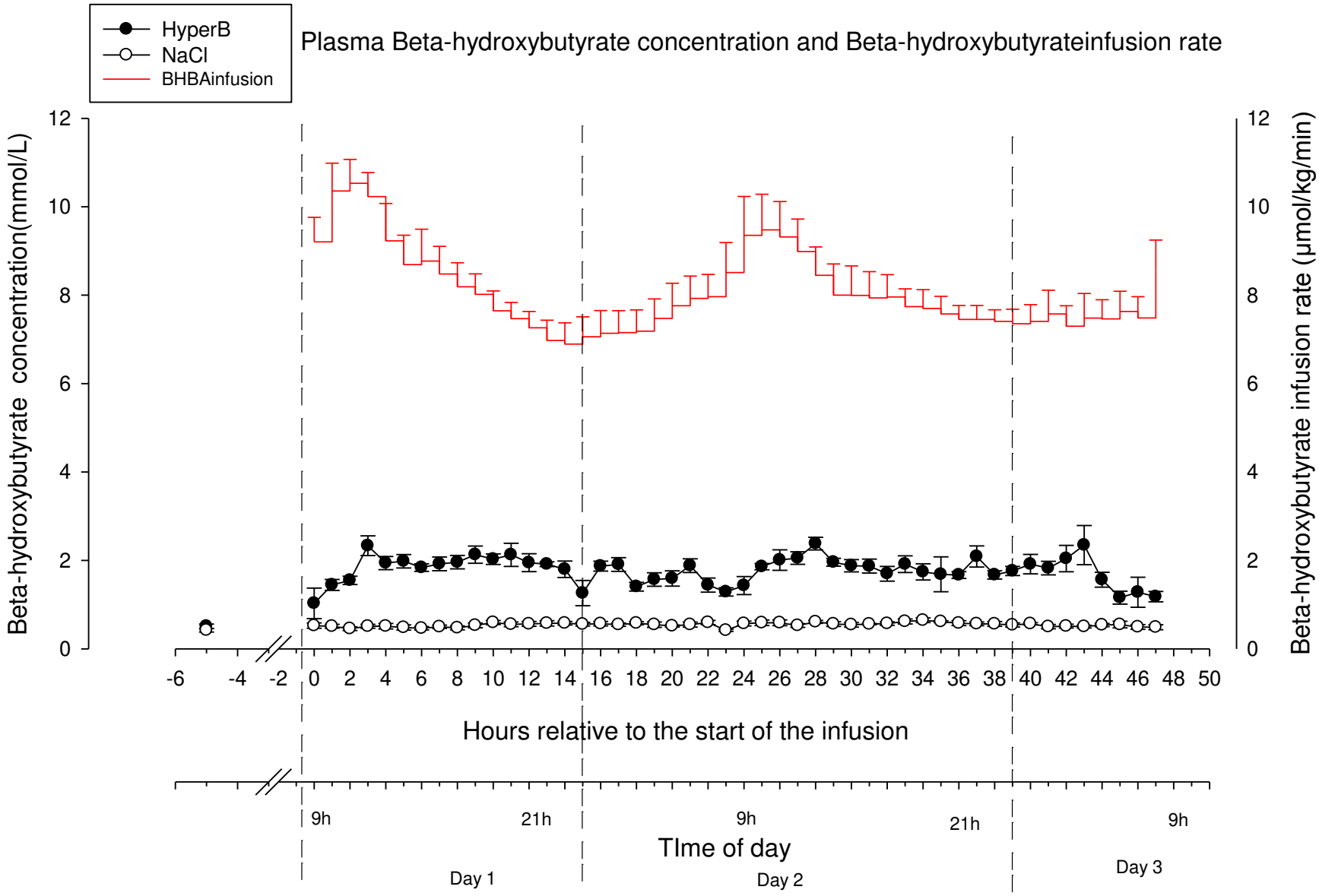
Figure legends

Figure 1. Beta-hydroxybutyrate (BHBA) infusion rate during 48 h infusion in BHBA cows (HyperB). Plasma BHBA concentration in control cows and cows infused with BHBA before the start of the infusion (d0) and during 48 h infusion. Values represent Mean ± SEM.

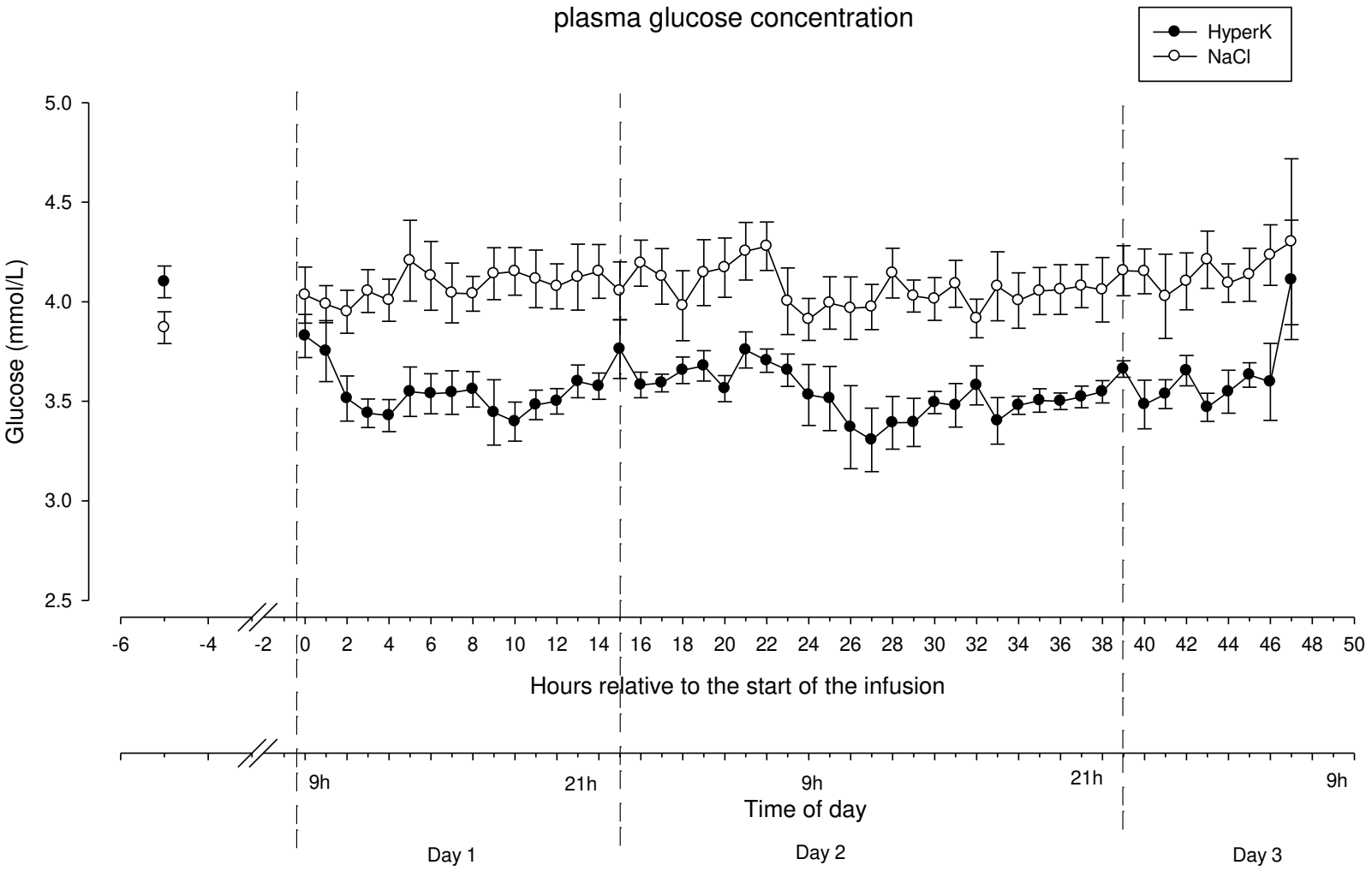
Figure 2. Plasma glucose concentration in control cows and cows infused with BHBA before the start of the infusion (d0) and during 48 h infusion. Values represent Mean ± SEM.

Figure 3. Plasma glucagon concentration in control cows and cows infused with BHBA before the start of the infusion (d0) and during 48 h infusion. Values represent Mean ± SEM.

Figure 4. Plasma insulin concentration in control cows and cows infused with BHBA before the start of the infusion (d0) and during 48 h infusion. Values represent Mean ± SEM.



JDS-12-6224, Figure 1



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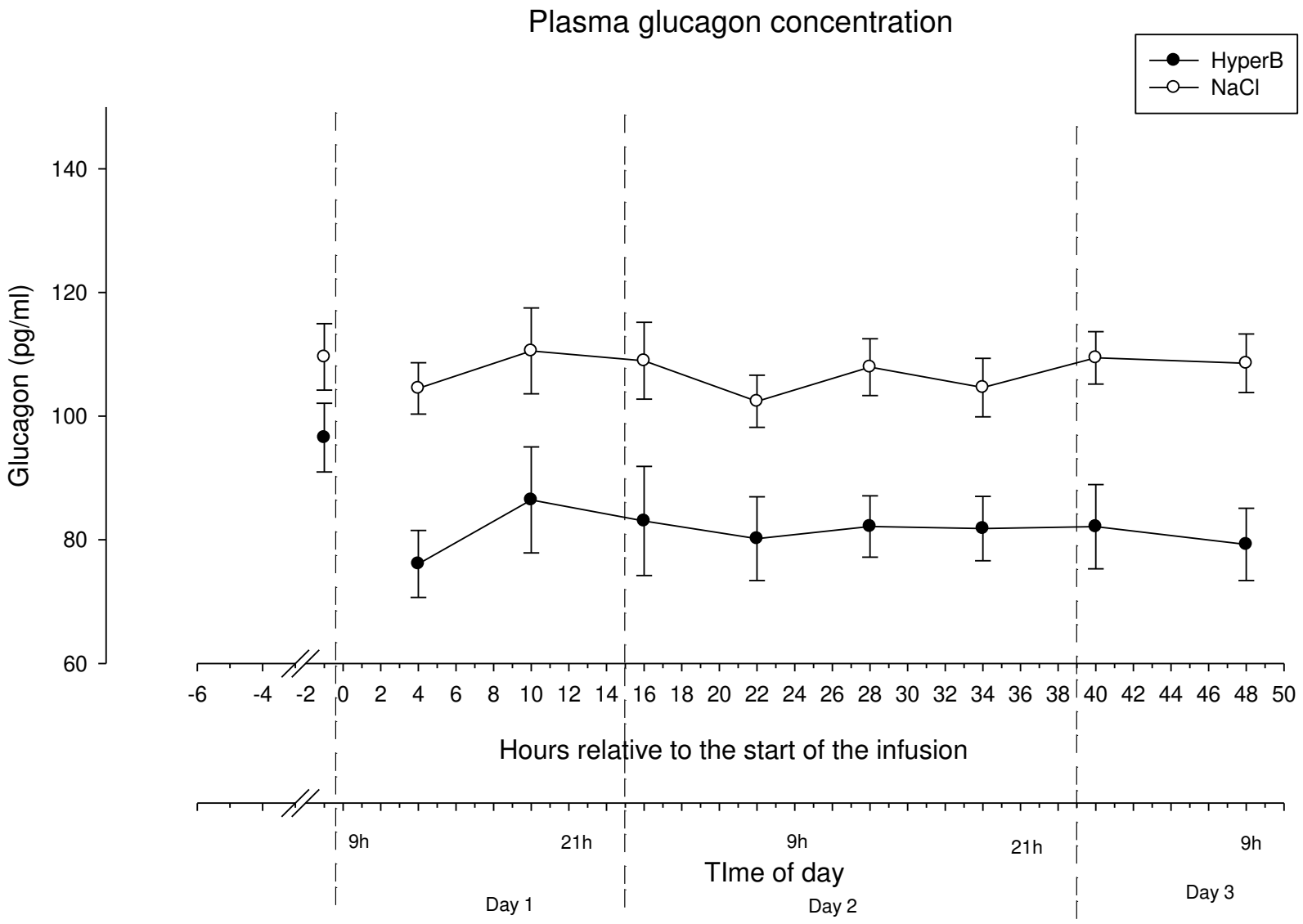

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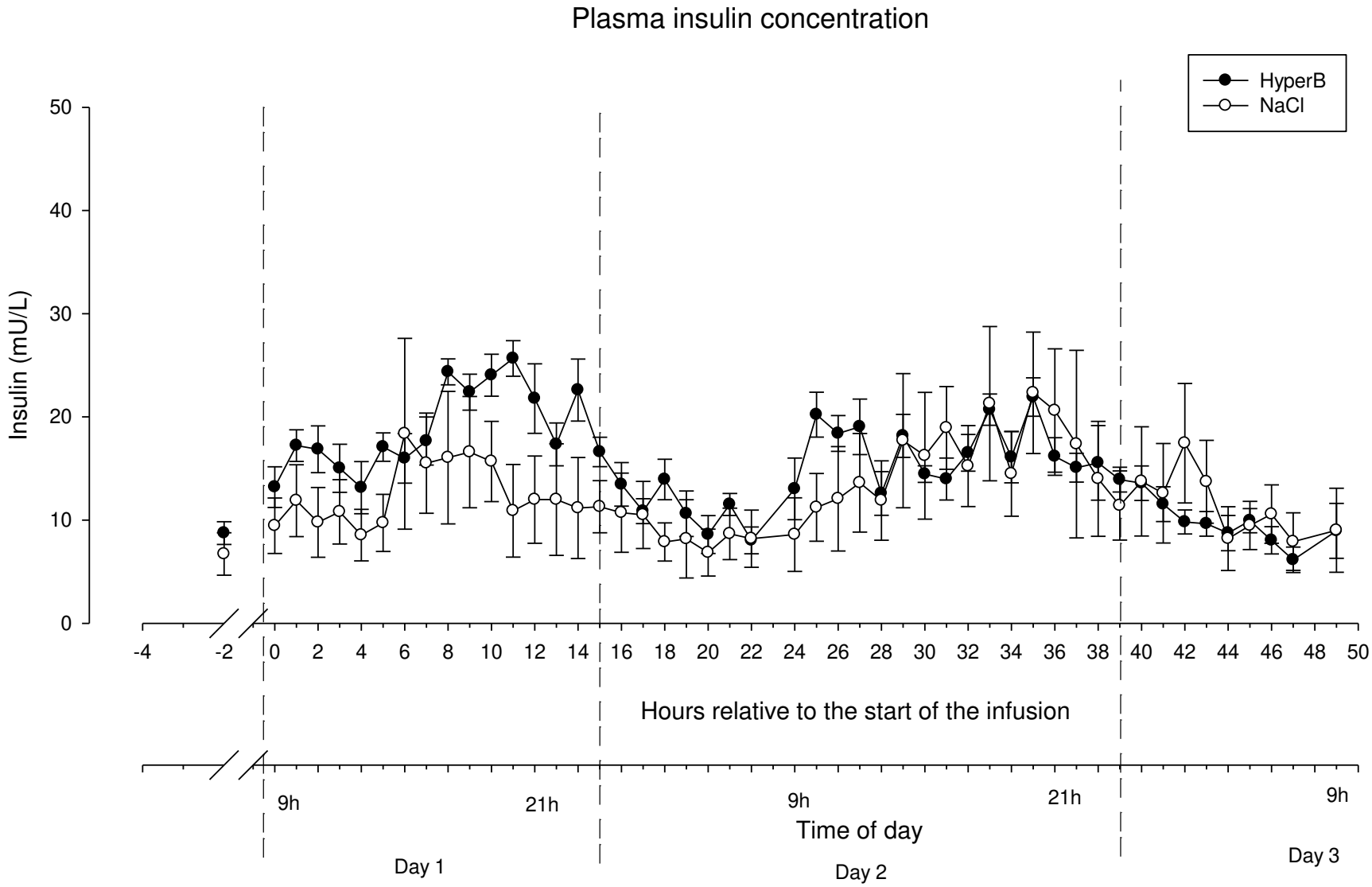

JDS-12-6224, Figure 4

Table 1. Polymerase chain reaction primer information (for = forward, rev = reverse), annealing temperature and the PCR product length

Gene[1]		Sequence 5'-3'	Gene Bank accession no.	Annealing temperature (°C)	Length, bp
Gluconeogenesis related variables					
G6PC	for	GCCAACCTACAGATTTCGGTG	NM-001076124	58	140
	rev	CAATGCCTGACAAGACTCCAG			
PC	for	ATCTCCTACACGGGTGACGT	NM_177946	60	214
	rev	TGTCGTGGGTGTGGATGTGCA			
PEPCKm	for	TACGAGGCCTTCAACTGGCGT	XM_583200	60	365
	rev	AGATCCAAGGCGCCTTCCTTA			
Phosphofructokinase ,liver and lactate dehydrogenase					
LDHA	for	CGGTTCACAAACAAGTGGTTG	NM-174099	60	166
	rev	GAGACCCTTAATCATGGTGG			
LDHB	for	CAGCATTCTGGGAAAGTCTC	NM-174100	60	141
	rev	GAGTAATCTTTGTCTGCCAC			
PFKL	for	TTGACTGCAGGACCAACGTC	NM-001080244	60	120
	rev	GCAGCTTCTCCGACATCCAG			
Glucagon receptor, glycogen synthase, protein kinase, AMP- activated alpha 1					
GCGR	for	TCCCAGTGTCAGATGGATGA	NM-001192414	60	207
	rev	CTTGAGCATGAAGGACACGA			
GYS2	for	AAACTTCCTGCTGAGGATGC	NM-001192905	58	138
	rev	GAATGTGCAGTGTCCCATAG			
PRKAA1	for	AAACTGTACCAGGTCATCAG	NM-001109802	58	326
	rev	GAAATTACTTCTGGTGCAGC			
Pyruvate dehydrogenase complex variables					
DBT	for	GTCGCCTTGCAATGGAAAAC	NM-173905	60	248
	rev	GTGAAAGCCTTTCACGGGTTC			
DLD	for	TGGTCAAGAGAGGCCATTTC	NM-001206170	60	16
	rev	AACTGGGCAGCTTTAATAGC			
PDHA1	for	ACACAGCATGAGTGATCCTG	NM-001101046	60	127
	rev	CTTCAACACTGGCGAGATTG			
Fatty acid and cholesterol synthesis related variables					
ACOAC	for	CTCTTCCGACAGGTTCAAGC	AJ-132890	61	248
	rev	ACCATCCTGGCAAGTTTCAC			
FASN	for	CTGAGTCGGAGAACCTGGAG	NM_001012669	63	232
	rev	ACAATGGCCTCGTAGGTGAC			
HMGCS1	for	TGTACGGCTCCCTGGCTTCTG	BC-102850	60	313
	rev	CATGTTCCTTCGAAGAGGGAATC			
Fatty acid oxidation related variables					
ACSL1	for	TGACTGTTGCTGGAGACTGG	NM-001076085	61	199
	rev	CAGCCGTCTTTATCCAGAGC			
CPT1	for	CAAAACCATGTTGTACAGCTTCCA	BF039285	54	111
	rev	GCTTCCTTCATCAGAGGCTTCA			
CPT2	for	CAGCCTGCCCAGGCTGCCTAT	BC105423	60	163
	rev	AGTGACCAGCTGCTCATGCA			

Citrate synthase, and ketogenesis related variables					
CS	for	TGGACATGATGTATGGTGG	BC-114138	60	217
	rev	AGCCAAGATACCTGTTCCTC			
HMGCS2	for	TCTGGCCCATCACTCTGCC	NM-001045883	60	126
	rev	AGTGGGGAGCCTGGAGAAGC			

[1]ACoAC = acetyl-CoA carboxylase; ACSL1 = acyl-CoA synthetase long-chain; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; CS = citrate synthase; DBT = dihydrolipoamide branched chain transacylase E2; DLD = Dihydrolipoamide dehydrogenase; FASN= fatty acid synthase; G6PC = glucose-6-phosphatase; GCGR= glucagon receptor; GYS2= glycogen synthase 2; HMGCS1 = 3- hydroxyl-3 – methylglutaryl-coenzyme A synthase 1; HMGCS2 = 3- hydroxyl-3 – methylglutaryl-coenzyme A synthase 2 ; LDHA = lactate dehydrogenase A; LDHB = lactate dehydrogenase B; PC = pyruvate carboxylase; PDHA1 = pyruvatedehydrogenase (lipomide) alpha 1; PEPCKm = mitochondrial phosphoenolpyruvate carboxykinase; PFKL = phosphofructokinase,liver; PPKA1= protein kinase, AMP- activated alpha 1.

Table 2. Least square means ± SEM of area under the curve (AUC) Plasma variables concentration in dairy cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl) on day 2.

Variable [1]	Group[2]	LSMeans ± SEM day 2	ANOVA (*P*-Value, group)
Glucose, mmol/L	HyperB	3.47 ± 0.11	< 0.01
	NaCl	4.11 ± 0.08	
INS, mU/L	HyperB	12.7 ± 1.4	0.54
	NaCl	13.9 ± 1.1	
BHBA, mmol/L	HyperB	1.74 ± 0.02	< 0.001
	NaCl	0.59 ± 0.02	
NEFA, mmol/L	HyperB	0.06 ± 0.03	0.51
	NaCl	0.09 ± 0.02	
Urea, mmol/L	HyperB	3.77 ± 0.31	0.63
	NaCl	3.97 ± 0.24	
Glucagon, pg/ml	HyperB	97.4 ± 3.3	< 0.05
	NaCl	107.7 ± 2.6	
IGF-1, ng/mL	HyperB	90 ± 4.5	0.11
	NaCl	80 ± 3.6	
TG, mmol/L	HyperB	0.15 ± 0.01	0.58
	NaCl	0.14 ± 0.01	
Cortisol, ng/mL	HyperB	2.41 ± 0.55	0.78
	NaCl	2.62 ± 0.43	

[1]BHBA= Beta-hydroxybutyrate; INS= Insulin; NEFA= non-esterified fatty acids; IGF-1= Insulin Growth Factor-1; TG= Triglyceride

[2] HyperB= Hyper beta hydroxybutyrate group; NaCl= group of cows receiving physiological saline solution

Table 3. Milk yield, ECM, DMI, and Energy balance of dairy cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl) before the start of the infusion (d0) and difference (delta: d2-d0) between before and after 48h infusion(d2). Values represent Mean ± SEM.

Variable [1]	Group[2]	d0	Delta d2-d0	ANOVA (*P*-Value, group)
Milk yield, Kg/day	HyperB	23.31 ± 0.78	-3.30 ± 1.43*	0.3
	NaCl	21.78 ± 2.53	-1.76 ± 0.68	
ECM, Kg/day	HyperB	21.00 ± 0.28	-1.58 ± 0.76	0.79
	NaCl	21.95 ± 2.58	-1.21 ± 0.85	
DMI, Kg/day	HyperB	19.82 ± 0.66	-0.80 ± 1.22	0.76
	NaCl	18.33 ± 1.28	-0.38 ± 0.76	
EB, MJ/d	HyperB	15.04 ± 2.18	-1.04 ± 8.67	0.77
	NaCl	1.67 ± 4.90	1.72 ± 4.90	

[1] DMI= dry matter intake; ECM= energy corrected milk; EB= energy balance;

[2] HyperB= Hyper beta-hydroxybutyrate group; NaCl= cow group receiving physiological saline solution

*Delta is different from 0 ($P < 0.05$)

Table 4. Plasma variables concentrations in dairy cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl) before the start of the infusion (d0) and difference (delta: d2-d0) between before and after 48h infusion (d2). Values represent Mean ± SEM.

Variable [1]	Group[2]	d0	Delta d2-d0	ANOVA (*P*-Value, group)
Glucose, mmol/L	HyperB	4.10 ± 0.08	-0.46 ± 0.18*	< 0.05
	NaCl	3.87 ± 0.08	0.10 ± 0.14	
INS, mU/L	HyperB	8.74 ± 0.78	-1.56 ± 0.92	0.30
	NaCl	6.78 ± 1.62	0.14 ± 1.08	
BHBA, mmol/L	HyperB	0.51 ± 0.05	0.74 ± 0.15*	< 0.001
	NaCl	0.42 ± 0.04	0.09 ± 0.05	
NEFA, mmol/L	HyperB	0.11 ± 0.02	-0.02 ± 0.02	0.7
	NaCl	0.16 ± 0.03	-0.04 ± 0.04	
Urea, mmol/L	HyperB	4.73 ± 0.34	-1.25 ± 0.3*	0.73
	NaCl	5.0 ± 0.26	-1.05 ± 0.4*	
IGF-1, ng/mL	HyperB	91 ± 6.1	-6.65 ± 2.5	0.55
	NaCl	100 ± 10.0	-9.87 ± 3.7*	
Glucagon, pg/ml	HyperB	115.2 ± 19.1	-17.3 ± 1.3*	0.06
	NaCl	109.6 ± 5.4	-1.0 ± 6.1	
TG, mmol/L	HyperB	0.18 ± 0.01	-0.02 ± 0.01	0.91
	NaCl	0.16 ± 0.01	-0.02 ± 0.01*	
Cortisol, ng/mL	HyperB	5.74 ± 1.4	-2.37 ± 1.9	0.67
	NaCl	2.92 ± 0.8	-1.56 ± 0.9	

[1]BHBA= Beta-hydroxybutyrate; INS= Insulin; NEFA= non-esterified fatty acids; IGF-1= Insulin Growth Factor-1; TG= Triglyceride

[2] HyperB= Hyper beta-hydroxybutyrate group; NaCl= cow group receiving physiological saline solution

[*]Delta is different from 0 ($P < 0.05$)

Table 5. Liver mRNA expression in dairy cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl) before the start of the infusion (d0) and difference (delta: d2-d0) between before and after 48h infusion (d2). Values represent Mean ± SEM.

Variable [1]	Group [2]	d0	Delta d2-d0	ANOVA (*P*-Value, group)
Gluconeogenesis related variables				
G6PC	HyperB	22.89 ± 0.46	-0.57 ± 0.50	0.44
	NaCl	21.05 ± 0.49	-0.18 ± 0.24	
PC	HyperB	17.42 ± 0.22	0.43 ± 0.08	0.87
	NaCl	17.57 ± 0.13	0.48 ± 0.23*	
PEPCKm	HyperB	14.60 ± 0.43	0.46 ± 0.29	0.18
	NaCl	14.85 ± 0.20	-0.21 ± 0.32	
Phosphofructokinase ,liver and lactate dehydrogenase				
LDHA	HyperB	16.58 ± 0.22	-0.58 ± 0.32	0.80
	NaCl	16.50 ± 0.30	-0.45 ± 0.34	
LDHB	HyperB	19.47 ± 0.32	0.01 ± 0.18	0.52
	NaCl	19.68 ± 0.38	-0.36 ± 0.45	
PFKL	HyperB	15.00 ± 0.25	0.35 ± 0.37	0.35
	NaCl	15.38 ± 0.21	-0.22 ± 0.40	
Glucagon receptor, glycogen synthase, protein kinase, AMP- activated alpha 1				
GCGR	HyperB	17.92 ± 0.19	-0.33 ± 0.24	0.91
	NaCl	16.98 ± 0.56	-0.24 ± 0.28	
GYS2	HyperB	19.03 ± 0.28	-0.90 ± 0.35	0.83
	NaCl	17.56 ± 0.49	-0.57 ± 0.29	
PRKAA1	HyperB	15.19 ± 0.22	-0.57 ± 0.30	0.08
	NaCl	14.11 ± 0.36	0.11 ± 0.21	
Pyruvate dehydrogenase complex variables				
DBT	HyperB	18.19 ± 0.18	-0.09 ± 0.23	0.90
	NaCl	17.64 ± 0.27	-0.21 ± 0.68	
DLD	HyperB	18.14 ± 0.17	-0.52 ± 0.31	0.84
	NaCl	17.90 ± 0.14	-0.38 ± 0.48	
PDHA1	HyperB	17.94 ± 0.14	-0.58 ± 0.46	0.53
	NaCl	18.09 ± 0.17	-1.03 ± 0.46*	
Fatty acid and cholesterol synthesis related variables				
ACOAC	HyperB	10.41 ± 0.18	-0.44 ± 0.34	0.64
	NaCl	8.66 ± 0.51	-0.74 ± 0.43	
FASN	HyperB	14.0 ± 0.4	-0.8 ± 0.3	0.82
	NaCl	14.0 ± 0.51	-0.9 ± 0.5	
HMGCS1	HyperB	20.95 ± 0.24	-1.61 ± 0.51*	0.69
	NaCl	18.84 ± 0.46	-1.24 ± 0.60*	
Fatty acid oxidation related variables				
ACSL	HyperB	19.76 ± 0.17	0.05 ± 0.19	0.65
	NaCl	18.87 ± 0.49	-0.07 ± 0.16	
CPT1	HyperB	14.82 ± 0.22	-0.28 ± 0.29	0.55
	NaCl	13.16 ± 0.36	-0.09 ± 0.16	
CPT2	HyperB	18.07 ± 0.12	-0.33 ± 0.10	0.54
	NaCl	17.19 ± 0.29	-0.46 ± 0.14	
Citrate synthase, and ketogenesis related variables				
CS	HyperB	14.48 ± 0.06	-0.26 ± 0.15	0.91
	NaCl	12.77 ± 0.40	-0.23 ± 0.20	
HMGCS2	HyperB	21.62 ± 0.33	-0.23 ± 0.33	0.77
	NaCl	21.14 ± 0.32	-0.10 ± 0.29	

[1]ACoAC = acetyl-CoA carboxylase; ACSL1 = acyl-CoA synthetase long-chain; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; CS = citrate synthase; DBT = dihydrolipoamide branched chain transacylase E2; DLD = Dihydrolipoamide dehydrogenase; FASN= fatty acid synthase; G6PC = glucose-6-phosphatase; GCGR= glucagon receptor; GYS2= glycogen synthase 2; HMGCS1 = 3- hydroxyl-3 – methylglutaryl-coenzyme A synthase 1; HMGCS2 = 3- hydroxyl-3 – methylglutaryl-coenzyme A synthase 2 ; LDHA = lactate dehydrogenase A; LDHB = lactate dehydrogenase B; PC = pyruvate carboxylase; PDHA1 = pyruvatedehydrogenase (lipomide) alpha 1; PEPCKm = mitochondrial phosphoenolpyruvate carboxykinase; PFKL = phosphofructokinase,liver; PPKA1= protein kinase, AMP- activated alpha 1.

[2] HyperB= Hyper beta-hydroxybutyrate group; NaCl= cow group receiving physiological saline solution

[*]Delta is different from 0 ($P < 0.05$)